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High CO_2 effects on growth and biometal contents in the pioneer species *Senna reticulata:* climate change predictions

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ABSTRACT

The aim of the present study consisted in evaluating the effects of CO_2 enrichment on the growth and biometal/nutrient content and accumulation in *Senna reticulata* germinated under two different carbon dioxide concentrations: atmospheric (360 mg L^{-1}) and elevated (720 mg L^{-1}). Biometal/nutrient determinations were performed on three different plant portions (leaflets, stem and root) using flame atomic absorption spectrometry. In general, the biometal and nutrient stoichiometries in roots were increased, probably due to reduced transpiration, and consequent biometal accumulation. An Artifical Neural Network analysis suggests that Mg, Na and Fe display the most different behavior when comparing plants germinated at atmospheric and elevated CO_2 conditions. Biomass and growth increases and certain elemental levels indicate that *S. reticulata* benefits from increased CO_2 levels, however some results indicate the contrary, making further studies in this context necessary, as these changes may lead to direct effects on food safety, crop yields, and phytoremediation efficiency.

1. Introduction

Carbon dioxide (CO_2) is one of the main gases that increases the greenhouse effect. Today, atmospheric CO_2 levels are in the order of 360 mg L^{-1}, although recent reports have indicated that levels have gone up to 400 mg L^{-1} in some areas, and it is estimated that this value will double to 720 mg L^{-1} in 50 years [1]. Because of this, it is imperative to evaluate the effects of high CO_2 concentrations in plants, since they may be severely affected by these global changes and both humans and animals depend on these organisms for survival [2,3].

Increased CO_2 concentrations have several beneficial effects; they provide more substrate available for photosythesis and, consequently, increase plant photosynthetic rates and carbon assimilation, decreasing photorespiration rates and dry matter production [4–6]. Changes to the composition of the plant itself may also occur in increased CO_2 conditions, since the requirement and stoichiometry of essential macro and micronutrients and trace elements important for plant metabolism can also be modified in these conditions [7–9]. For example, the activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), an enzyme involved in the first major step of carbon fixation, is directly dependent on Mg^{2+} for enzymatic activity, demonstrating the importance of essential macro- and trace elements in plant metabolism [10].

Senna reticulata (Willd.) H. S. Irwin & Barneby, is popularly known as a “pasture killer”, due to its high competition with other plants, growth ability and easy establishment in pastures and open areas [11]. Because of this, it is considered a plague and eliminated periodically by farmers in pasture areas in order to preserve grass cultivation [12,13]. This is a C3 species, meaning that it uses the C3 metabolic pathway for carbon fixation in photosynthesis, converting carbon dioxide from the air into 3-phosphoglycerate instead from malate, as occurs in plants that use the C4 metabolic pathway [14]. *Senna reticulata* shows the highest natural photosynthetic assimilation measured in lowland Amazonian trees, with values up to 30 mmol CO_2 m^{-2} s^{-1}, significantly higher than the typical values for tropical species (i.e. *Vitex cymosa* Beth., about 8 mmol CO_2 m^{-2} s^{-1}; *Nectandra amazonum* Nees, about 7 mmol CO_2 m^{-2} s^{-1}) [12], suggesting potential for high growth rates in elevated CO_2 conditions. In the long-term, elevated CO_2effects are mediated by several parameters, such as source-sink interactions within plants, temperature, land-use management practice, and, especially,

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resources, such as nutrients [15].

In this context, the present study aimed to evaluate increased CO_2 effects on the growth and stoichiometries of four macroelements (Na, Ca, K and Mg) and two trace elements (Mn and Fe) of *Senna reticulata* specimens germinated in atmospheric and elevated CO_2 concentrations in three different plant parts: leaflets, stem and root.

2. Methodology

2.1. Germination

The source of the seeds used in the germination experiments were adult *Senna reticulata* samples collected in the city of Belém, Pará state, Northern Brazil.

Seed scarification was conducted by wearing out the outside of the seeds with sandpaper. The seeds were then allocated in petri dishes with filter paper and vermiculite in a 3:2 ratio for germination and placed in a Bio-Oxygen Demand incubator at 29 °C for a 12 hs photoperiod.

After germination, the seedlings were removed from the incubator and transferred to soil-containing pots. A Hogland, iron-EDTA and a NaOH solution were added to each plant, to avoid nutrient availability deficiency, in order to asses only CO_2 effects with no nutrient differences between the groups. The Hogland solution was composed of $MnCl_2$: 0.362 g L^{-1}; $ZnSO_4$: 0.044 g L^{-1}; $CuSO_4$: 0.018 g L^{-1}; H_3BO_3: 0.572 g L^{-1}; KH_2PO_4: 27.20 g L^{-1}; $MgSO_4$: 98.60 g L^{-1} and $CaCl_2$: 247.02 g L^{-1}; the iron-EDTA solution was composed of $FeSO_4{\cdot}7H_2O$: 50 g L^{-1} and EDTA-Na: 6.64 g L^{-1}; and the NaOH solution was of 0.73 g L^{-1}. The pots were then filled with washed sand and vermiculite at a 3:2 ratio and placed into the incubators at atmospheric (360 mg L^{-1}) and elevated (720 mg L^{-1}) CO_2 concentrations.

The Hogland solution was added to the soil weekly and irrigation was conducted daily in the aerial portion of the plant. Extracting solutions were added only after irrigation, to avoid leaching from the superficial parts of the plants. The pots were permutated weekly inside the incubator, to ensure homogeneous solar availability for all specimens.

2.2. Growth measurements

At the end of the desired growth period of three months, leaf area, leaf mass, stem mass, root mass and total biomass measurements were conducted. Leaf areas were calculated with aid of a caliper (± 0.1 mm). Transpiration measurements were carried out using a LI-6400 infra-red gas analyzer (IRGA) (Li-Cor, USA), both during the day and at night. All analyses were performed in triplicate.

2.3. Carbon determinations

C analyses were carried out on a Perkin-Elmer 240 CHN analyzer after sample combustion.

2.4. Trace element analyses

Samples were dried in an oven at 100 °C until constant weight and ground with a mortar and pestle. Approximately 0.1 g of each individual plant portion (leaflets, roots and stems) were dissolved in a microwave oven with temperature increase steps from 0 °C to 80 °C, 80 °C to 150 °C and 150 to 280 °C, using ultrapure water (resistivity > 18.0 MΩ cm) and a mixture of distilled HNO_3 (Isofar, Rio de Janeiro, Brasil) and H_2O_2 (Synth, São Paulo, Brazil). After digestion, the samples were transferred to 25 mL flasks and made up to 25 mL with ultrapure water. K, Na, Ca, Mg, Fe and Mn were determined on a SpectrAA 55 flame atomic absorption spectrometer (Varian, USA). Instrumental conditions are displayed in Table 1.

Table 1
Instrumental atomic absorption spectrometer conditions. Lamp current was 10 mA for all elements.

Element	Slit	Wavelength (nm)	Suppressor	Gas
Ca	0.5	324.8	La_2O_3 (0.5865 g L^{-1})	N_2O_2/Air/C_2H_2
Mg	0.5	285.2		Air/C_2H_2
Na	0.5	589.0	–	Air/C_2H_2
K	1.0	766.5	–	Air/C_2H_2
Mn	0.5	324.8	–	Air/C_2H_2
Fe	0.2	248.3	$CaCO_3$ (0.630 g L^{-1})	Air/C_2H_2

In the present study, external analytical curves were used for all metal and metalloid determinations, using multielemental calibration solutions obtained by appropriate dilutions of a mixed standard solution (Merck IV, Merck, Germany). Precision was calculated by means of the relative standard deviation (RSD), which measures the repeatability of the method, the Limit of Detection and Limit of Quantification for each analyte, and the overall uncertainty of the measurements was determined for each element through Kragten's method, as recommended in the EURACHEM/CITAC Guide Section 8.2.5 and Appendix E [16,17]. These figures of merit are displayed in Table 2.

Table 2
Figures of merit. RSD – Relative standard deviation (%), LOD – Limit of Detection (mg g^{-1}), LOQ – Limit of Quantification (mg g^{-1}), R^2 – linear coefficients for the calibration curves for each element, U – Kragten's method for measurement uncertainty.

Element	RSD (%)	LOD	LOQ	R^2	U
Ca	1.32	0.033	0.111	0.998	0.83
Mg	3.23	0.024	0.080	0.999	0.16
Na	3.72	0.005	0.016	0.998	1.23
K	2.17	0.011	0.037	0.999	0.85
Mn	1.48	0.013	0.043	0.998	3.84
Fe	2.25	0.036	0.121	0.999	3.34

The accuracy of the analytical procedure was confirmed by the standard addition method for each element and the parallel analysis of a standard reference material (NIST SRM 1515 – Apple leaves – Table 3). All analyses were performed in triplicate.

Table 3
Certified and observed values using standard reference material NIST SRM 1515 – apple leaves. Values are shown as means ± standard deviation (mg g^{-1}). Ca, Mg and K values are expressed as g kg^{-1}, while Na, Mn and Fe values ares expressed as μg g^{-1}.

Element	Certified value	Found value	(%) Recovery
Ca	15.26 ± 0.00	14.85 ± 0.18	97.31 ± 1.18
Mg	2.71 ± 0.00	2.12 ± 0.08	78.23 ± 2.95
Na	24.40 ± 1.20	12.75 ± 1.03	52.25 ± 4.22
K	16.10 ± 0.00	11.64 ± 0.20	72.30 ± 1.24
Mn	54.00 ± 3.00	58.50 ± 3.86	108.33 ± 7.15
Fe	83.00 ± 5.00	60.25 ± 4.64	72.59 ± 5.60

2.5. Data analysis

Multivariate analysis techniques (correlation analysis and cluster analysis) and Artificial Neural Networks (ANN) were used to evaluate the influence of CO_2 on metal nutrient behavior in the different plant parts.

2.5.1. Multivariate analysis techniques

The cluster analysis method was composed of a k-means analysis, that aims to construct a group of k groups from a given data mass (m), in a way that each unit belongs to only one group [18].

2.5.2. ANN analysis

The ANN adopted in the present study was a direct multilayer ANN with a backpropagation training algorithm. The topology consisted of an input layer with six neurons, one for each input variable (the trace elements) two intermediate layers with five and four neurons and two output layers with three neurons. The response variables were the plant parts and the CO_2 germination concentrations. A Standardized Rescaling Method for Covariates and Normalized Rescaling Method for Scale Dependents were used. The ANN training used the following parameters [19]:

1 The weights (synapses) were randomly initialized in the interval [−1 1];
2 The learning rate used in the backpropagation training algorithm was 0.005;
3 The sigmoid transfer function was used in all neurons of the intermediate and output layers of the network;
4 The stop criterion of training was established by the network approximation error (mean square error), in the order of 10^{-6};
5 The activation function was sigmoidal with standardized variables.

3. Results

3.1. Quality assurance and laboratory sample representativeness

The parallel analysis of the standard reference material NIST SRM 1515 – apple leaves showed no statistically significant difference (ANOVA; $p < 0.05$) between certified and observed values (Table 2). Thus, the digestion procedure was considered adequate for the present study. Although Na recovery was low, repeatability showed a coefficient of variation lower than 5%.

3.2. Biomass, transpiration and carbon content

Seedlings exposed to increased CO_2 conditions showed significant biomass increases compared to those exposed to atmospheric CO_2 concentrations, while transpiration rates were significantly reduced in elevated CO_2 conditions. A small decrease in carbon content in leaves was observed for plants exposed to elevated CO_2, while a small increase was observed in stems (Table 4).

3.3. Metal nutrient concentrations

Root trace nutrient concentrations were higher than stem and leaflets for all elements, except for Fe. Leaflets showed significant reductions (52.56%) in Na levels when exposed to 720 mg L^{-1} of CO_2 concentrations when compared to the 360 mg L^{-1} exposure, while both root and stem showed significant increases of this element at 720 mg L^{-1}, of 234.74% and 168.34%, respectively. K showed the same behavior as Na, although in much smaller scale, with a 15.40% reduction of this element in plants exposed to 720 mg L^{-1}, while root and stem showed small increases, of 35.41% and 3.17%, respectively. Ca concentrations, on the other hand, showed small decreases in leaflets exposed to 720 mg L^{-1} CO_2 (1.10%), while roots showed a 56.02% increase. Stems showed a 10.21% decrease in Ca concentrations. Mg was the only element that showed increased values in all three plant sections germinated at 720 mg L^{-1}, increasing 15.06% for leaflets, 111.45% for roots and 6.15% for stems. Fe, on the other hand, showed the opposite behavior, with decreased concentrations in all three plant compartments, of 1.96%, 19.16% and 41.72% for leaflets, roots and stems, respectively. Mn concentrations decreased in leaflets exposed to 720 mg L^{-1} CO2 (26.65%), while roots showed a 5.10% increase and stems showed a 19.38% decrease. Results are displayed in Figs. 1 and 2.

3.4. Factor analysis and ANN data analysis

3.4.1. Factor analysis

Since the factor analysis is based on the correlations between the variables, a correlation matrix between these variables must be plotted and examined, in order to verify the existence of significant Pearson coefficients values to justify the use of this technique. Variables that exhibit non-significant correlations with the other variables show low commonalities and should, therefore, not be a part of the analysis [20]. The correlations between the metal concentrations in each plant portion and the different CO_2 conditions were analyzed. Table 5 shows the correlation matrix with the significant Pearson correlation coefficients shown in bold, considering the bilateral test at a significance level of $\alpha = 5\%$.

Table 4
Biometric and transpiration data for *Senna reticulata* germinated at atmospheric (360 ppm) and elevated CO_2 (720 ppm) concentrations. Data is shown as means ± standard deviation (dry weight).

CO_2 Condition	Leaf area (mm^2)	Leaflet (g)	Stem (g)	Root (g)	Whole plant (g)	Transpiration rate (mmol)
360 ppm	2.32 ± 0.16	0.62 ± 0.04	0.97 ± 0.28	0.86 ± 0.21	2.46 ± 0.44	5.74 ± 0.71
720 ppm	3.94 ± 0.15	1.29 ± 0.16	2.40 ± 0.35	1.80 ± 0.31	5.50 ± 0.71	3.14 ± 0.26

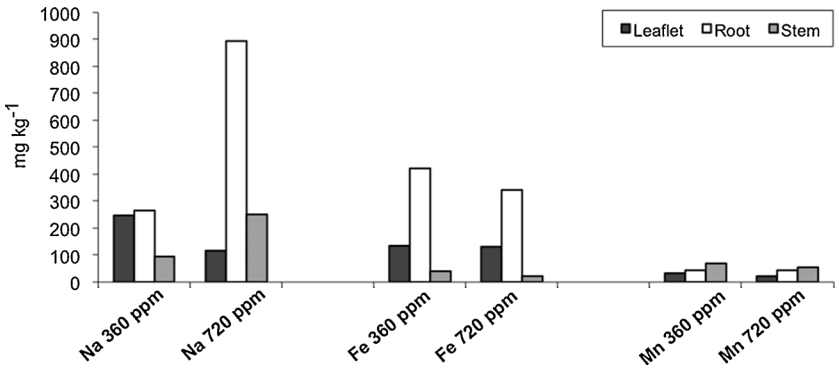

Fig. 1. Na, Fe and Mn concentrations in atmospheric (360 ppm) and elevated (720 ppm) in leaflets, stem and roots of *S. reticulata*.

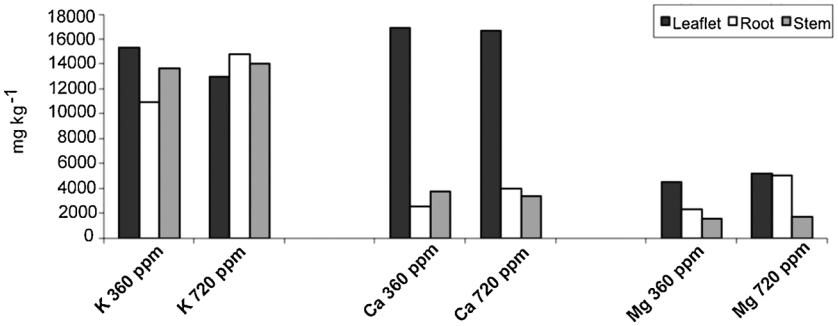

Fig. 2. K, Ca and Mg concentrations in atmospheric (360 ppm) and elevated (720 ppm) in leaflets, stem and roots of *S. reticulata*.

Table 5
Correlation matrix between the trace element concentrations in each plant portion and the different CO_2 concentrations used in *S. reticulata* seed germination.

360 ppm							720 ppm					
	Na	K	Ca	Mg	Mn	Fe		Na	K	Ca	Mg	Mn	Fe
Leaflets													
Na	1	**0.72**	**0.48**	−0.21	−0.46	−0.03	Na	1	**−0.46**	−0.37	−0.26	−0.11	**−0.49**
K	**0.72**	1	0.37	−0.23	**−0.56**	−0.09	K	−0.46	1	**0.41**	0.23	0.36	0.08
Ca	0.48	0.37	1	−0.25	−0.16	0.3	Ca	−0.37	**0.41**	1	**0.44**	**0.48**	−0.2
Mg	−0.21	−0.23	−0.25	1	−0.01	**−0.5**	Mg	−0.26	0.23	**0.44**	1	0.33	**−0.57**
Mn	**−0.46**	**−0.56**	−0.16	−0.01	1	−0.21	Mn	−0.11	0.36	**0.48**	0.33	1	−0.25
Fe	−0.03	−0.09	0.3	**−0.5**	−0.21	1	Fe	**−0.49**	0.08	−0.2	**−0.57**	−0.25	1
Stem													
Na	1	**0.66**	0.37	**0.81**	0.03	**0.75**	Na	1	0.05	0.17	0.07	−0.27	**−0.46**
K	0.66	1	**0.4**	**0.59**	0.17	**0.77**	K	0.05	1	−0.3	0.15	**0.49**	0.08
Ca	0.37	**0.4**	1	0.11	0.03	**0.51**	Ca	0.17	−0.3	1	−0.31	−0.04	−0.22
Mg	0.81	**0.59**	0.11	1	−0.03	**0.53**	Mg	0.07	0.15	−0.31	1	−0.07	0.35
Mn	0.03	0.17	0.03	−0.03	1	−0.1	Mn	−0.27	**0.49**	−0.04	−0.07	1	0.22
Fe	**0.75**	**0.77**	**0.51**	**0.53**	−0.1	1	Fe	−0.46	0.08	−0.22	0.35	0.22	1
Root													
Na	1	−0.26	0.33	−0.35	−0.34	0.06	Na	1	−0.36	**−0.73**	0.21	−0.06	**0.43**
K	−0.26	1	**−0.52**	0.04	0.09	**−0.68**	K	−0.36	1	**0.47**	**−0.54**	0.1	−0.2
Ca	0.33	**−0.52**	1	0.34	−0.25	**0.5**	Ca	**−0.73**	**0.47**	1	**−0.45**	−0.27	**−0.5**
Mg	−0.35	0.04	0.34	1	**−0.41**	0.08	Mg	0.21	**−0.54**	**−0.45**	1	**0.48**	**0.58**
Mn	−0.34	0.09	−0.25	**−0.41**	1	−0.17	Mn	−0.06	0.1	−0.27	**0.48**	1	0.32
Fe	0.06	**−0.68**	**0.5**	0.08	−0.17	1	Fe	**0.43**	−0.2	**−0.5**	**0.58**	0.32	1

Significant values are in bold

3.4.1.1. PCA analysis. After plotting the correlation matrix, a verification that the factor analysis pre-requisites were not violated was conducted, by the Bartlett's test of sphericity, the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and commonality assessments. The analysis showed a significant p (p = 0.000) in the Bartlett's test of sphericity, a KMO above 0.50 (0.75 for 360 mg L^{-1} and 0. 75 for 720 mg L^{-1}, respectively) and commonalities with values above 0.50, all non-violations of PCA pre-requisites. Thus, the Principal Component Analysis (PCA) with Varimax orthogonal rotation of the axis and Kaiser normalization was deemed appropriate and applied.

At the 360 mg L^{-1} CO_2 concentration, the PCA analysis showed 2 factors accounting for 99.00% of the total variability: Factor 1 (F1) corresponded to 68.18% of total variability and includes all metals, except Mg, while Factor 2 (F2) corresponded to 29.82% of the total variability and includes Mg (Table 6).

At the 720 mg L^{-1} CO_2 concentration two factors were also obtained, accounting for 99.15% of the total variability: Factor 1 (F1) corresponded to 51.17% of total variability and includes all metals, except Ca, Mg and Mn, while Factor 2 (F2) corresponded to 47.98% of the total variability and includes Na, K and Fe (Table 7).

The score graphs for each CO_2 concentration are displayed in Fig. 3.

The k-means classification technique was used in order to classify the trace metal concentrations in the 360 and 720 mg L^{-1} CO_2 germinations. Based on the p values, all metals were responsible for the formation of three clusters, corresponding to the three parts of the plant, at a significance level of $\alpha = 5\%$. 100% classification was obtained with the final centroids of the three plant parts for both germination situations.

Table 6
Metals in each factor and their commonalities at 360 ppm CO_2.

Metal	Commonalities	F_1	F_2
Na	0.99	**−0.83**	0.55
K	0.99	**0.73**	0.68
Ca	0.99	**0.99**	0.05
Mg	0.99	−0.10	**0.99**
Mn	0.99	**0.97**	−0.14
Fe	1.00	**−0.99**	−0.13
% of Variance		68.18	29.82
% Cumulative		68.18	99.00

Significant values are in bold

Table 7
Metals in each factor and their commonalities at 720 ppm CO_2.

Metal	Commonalities	F_1	F_2
Na	1	−0.23	**0.97**
K	0.98	−0.64	**0.76**
Ca	0.99	**0.91**	−0.4
Mg	1	**0.85**	0.53
Mn	0.98	**−0.99**	0.02
Fe	1	0.27	**0.96**
% of Variance		51.17	47.98
% Cumulative		51.17	99.15

Significant values are in bold

Table 8
Independent Variable Importance of each trace element using ANN Analysis.

Independent Variable Importance		
Element	Importance	Normalized Importance
Na	0.226	96.90%
K	0.057	24.40%
Ca	0.171	73.30%
Mg	0.104	44.50%
Mn	0.233	100.00%
Fe	0.208	89.20%

3.4.2. ANN analysis

The response variables of the ANN analysis were the plant parts and the different CO_2 germination concentrations (Fig. 4).

This ANN analysis resulted in the following trace elements importance, seen in Table 8.

4. Discussion

4.1. Biomass, transpiration and carbon content

All *Senna reticulata* portions (roots, stem and leaflets) exposed to elevated CO_2 levels showed significant biomass increases, in accordance to previous studies with other C3 plants species exposed to CO_2 enrichment [21–23]. Previous experiments [24] with *Senna alata*, a very similar species to *Senna reticulata,* for example, showed 60% increase in total plant biomass. In the present study, roots showed the highest increases in nutrient concentrations, followed by stems and leaflets. This corroborates several studies that observed a major increase in root biomass in elevated CO_2 conditions, since these conditions generally increase plant carbon allocation to belowground processes, which then facilitates root growth [25–27]. Thus, plant responses to increased CO_2 levels are directly influenced by the size and activity of belowground structures [28] and roots are responsible for

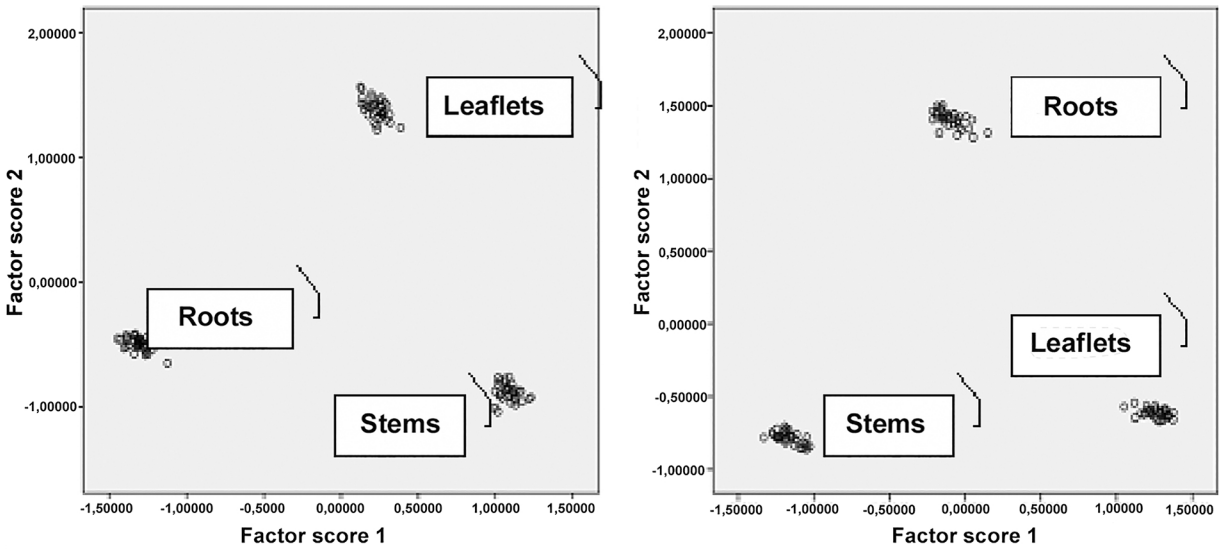

Fig. 3. Score graphs for each CO_2 concentration.

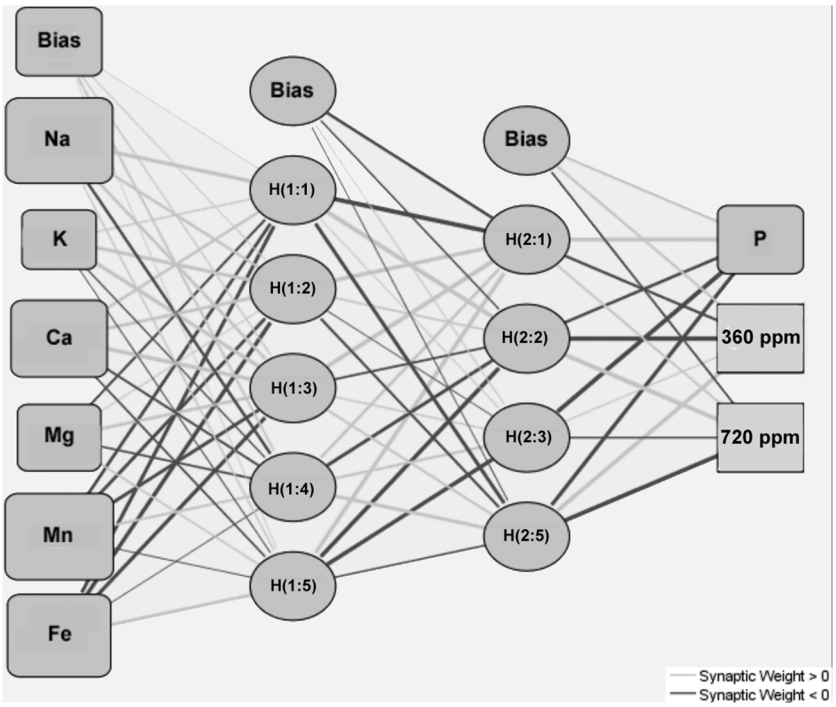

Fig. 4. ANN of the present study.

most of the nutrient uptake in several plant species [29].

Leaf transpiration rates in the present study were significantly decreased in plants germinated in elevated CO_2 conditions, as reported previously for C3 plants [30,31], and also for the similar species *S. alata* [24]. Since one of the purposes of transpiration is to move minerals from plant roots to other plant portions, these significantly lower transpiration rates in higher CO_2 concentrations could be partially responsible for the differences in nutrient accumulation and apportionment in the different plant portions, which will be discussed ahead.

4.2. Nutrient apportionment and accumulation

The nutrients analyzed in the present study are involved in many important plant physiological processes. K, for example, is a co-factor of many enzymes and necessary for regulatory processes and syntheses, such as protein biosynthesis. Ca participates in regulatory functions, plays a role in cell wall structure, stabilizes membranes and controls movements. Mg is a clorophyll component, a counter ion of ATP and extremely important for protein biosynthesis. Fe is necessary for clorophyll synthesis, and is required for the functioning of a range of enzymes, especially those involved in oxidation and reduction processes, such as cytochromes and ferredoxin. Mn is also a component of proteins and aids in chlorophyll synthesis [32].

Increased atmospheric carbon dioxide has been known to affect the biological storage and stoichiometry of trace metal nutrients and plant nutrient requirements [7,33]. However, there is no consensus regarding metal nutrient apportionment and accumulation in plants under elevated CO_2 conditions. For example, some studies report that generally trace elements in C3 plants are found to be lower in elevated CO_2 situations, due to a dilution effect caused by the characteristic increase in biomass at high CO_2 levels [34,35], while other reports observe the contrary [36,37].

In the present study, the effects of elevated CO_2 on *S. reticulata* nutrient concentrations were variable in each plant portion; some elements showed significant increases under elevated CO_2 conditions, others showed small decreases and, in some cases no significant differences were observed. CO_2 enrichment, therefore, does seem to affect micro and macronutrient uptake and their apportionment to different plant parts in *Senna reticulata*, but no unique trend was distinguishable, with the exception of increases in all the three plant portions for Mg and decreases for Fe.

In accordance to studies that indicate that plant responses to increased CO_2 levels are directly influenced by the size and activity of belowground structures [28] and that roots are responsible for most of the nutrient uptake in several plant species [29], root trace nutrient concentrations in the present study were higher than stem and leaflet concentrations for all elements, except for Fe. Higher concentrations in leaflets were observed for Ca and Mg only in enriched CO_2 conditions, while stem concentrations were higher in enriched CO_2 conditions for Na and K only. The other nutrients showed decreases in these plant portions, consistent with the "nutrient dilution effect" caused by enrichment in CO_2 levels.

Calcium is especially dependent on transpiration in most plant species (Marschner, 1995), and, since elevated CO_2 levels reduce leaf level transpiration in C3 plants, this may cause significant reductions in Ca uptake, as seen previously in wheat plants [38]. On the other hand, other studies have observed significantly reduced Ca concentrations in plant portions (i.e. leaves) of CO_2 enriched trees [39]. In the present study, Ca showed only small non-significant decreases in leaflet (1.10%) and stem concentrations (10.21%) and a significant increase in root concentrations (56.02%). Thus, elevated CO_2 concentrations seem to significantly influence Ca uptake and mobility in *S. reticulata* roots only.

K concentrations in *S. reticulata* were also variable between plant portions, with significant increases in the root (35.41%), non-significant increases in the stem (3.17%) and a significant reduction (15.40%) in leaflet concentrations. The latter reduction is in accordance to previous studies that have also shown significant K reductions in the leaves of CO_2 enriched trees [39]. However, other studies have observed higher leaf K concentrations in plants grown under elevated CO_2.

Mg, following the same behavior as Ca and K in previous studies, has also been shown to reduce significantly in leaves of CO_2 enriched trees [39]. This element is an essential component of the RuBisCo enzyme, which is Mg dependent in C3 plants, and high CO_2 levels have been shown to cause reductions in RuBisCo quantity and/or decreases in its activation state [40–42] in several C3 species. However, Mg excess in C3 plants has been shown to increase RuCisCo activation, plant physiology and metabolism [43]. It is interesting that, in the present study, Mg was the only element that showed increased values in all three plant compartments germinated at 720 mg L^{-1}, increasing significantly in leaflets (15.06%), and roots (111.45%) and non-significantly in stems (6.15%). This apparent increased Mg availability to *S. reticulata* roots may directly counter RuBisCo reduction in elevated CO_2 conditions, perhaps leading to the opposite, favouring improved photosynthetic rates.

4.2.1. Nutrient ANN analysis

The ANN analysis indicated that Mn was the most important element (100%) in the present study followed by Na (96.90%) and Fe (89.20%) when comparing plants germinated in atmospheric and elevated CO_2 conditions, indicating these three elements showed the greatest differences between the two CO_2 conditions. Therefore, these 3 micronutrients and their importance to plant physiology shall be discussed in further detail.

4.2.1.1. Manganese (Mn). Mn showed significantly decreased concentrations in leaflets (26.65%) and stem (19.38%), and a small, non-significant, increase in roots (5.10%), in contrast with previous studies that have reported increased Mn in foliar parts of plants under CO_2 enrichment [7,33], but in accordance to other reports that observed reductions in Mn concentrations in leaves of CO_2 enriched plants [39]. Since this element interacts strongly with other elements, which may lead to Mn toxicity, atmospheric factors, i.e. increased CO_2 levels, which affect the uptake of these nutrients may have important interactions with Mn phytotoxicity. For example, when increasing atmospheric CO_2 leaf transpiration usually decreases, which is known to reduce the uptake of Ca and Mg to the roots, as well as causing changes in Ca and Mg apportionment to expanding leaves by changing xylem fluxes [25]. These effects have been postulated as resulting in the exacerbation of Mn inhibition of leaf metabolism of Ca and Mg. However, this does not seem to be the case in the present study, and *S. reticulata* does not seem to be at risk for this inhibition, since Ca showed a non-significant decrease (1.10%) in foliar concentrations, while Mg showed a 15.06% increase.

Mn is also essential to the photosystem II process, where it forms a Mn-Ca oxide complex, oxidizing water to form hydrogen ions and molecular oxygen providing the electrons for all of photosynthesis to occur. Since the amount of functional photosystem II reaction centers has been show to be compromised in plants that exhibit intermediate and low amounts of manganese-stabilizing proteins [44], the significantly decreased concentrations in leaflets and stem observed in the

present study may be of importance in this regard. Also, carboanhydrase activity of photosystem II proteins has been shown to be manganese dependent, which adds to the importance of this nutrient in plant physiology [45]. Reduced manganese has also been known to decrease carbon assimilation and stomatal closure [46], and the small decrease in carbon content in leaflets in the present study, although non-significant, may indicate a trend towards decreased carbon assimilation in *S. reticulata* in elevated CO_2 conditions. The small Mn increase in roots, although also non-significant, may also be of importance regarding CO_2 assimilation, since Mn-excess has been noted to decrease CO_2 assimilation, stomatal conductance and impairs the whole photosynthetic electron transport chain from the donor side of photosystem II up to the reduction of end acceptors of the photosystem I process, thus limiting the production of reducing equivalents, and hence the rate of CO_2 assimilation [47]. Therefore, the overall trend in *S. reticulata* exposed to high CO_2 conditions regarding Mn associated functions seem to indicate the potential for lower CO_2 assimilation in this species.

4.2.1.2. Sodium (Na). Studies indicate that C3 plants show no responses to experimental sodium additions (i.e. rice and salt-stressed *Brassica*) [48–50]. Not many studies regarding C3 plant species and their sodium requirements are available, although some authors indicate that under salt stresses, high Na concentrations can displace, and, therefore, interfere with Ca function in plants, leading to cell wall and membrane instability [51]. Most plants, however, use potassium, rather than sodium, as an important component of osmotic adjustment [52]. However, potassium and sodium ions compete for entry into plant cells [53], which makes the significantly higher Na concentrations in root and stem portions (234.74% and 168.34%, respectively) observed in the present study of interest regarding K/Na competition, with potentially significant effects on plant physiology regarding cell wall stability and plasma membrane integrity, as well as plant salt-tolerance [54,55].

Almost no literature regarding sodium concentrations in C3 plants exposed to higher CO_2 levels exists; in one study regarding leaves only, no statistically significant differences between Na concentrations was observed in plants germinated at high and atmospheric CO_2 concentrations [39]. This differs from our results, in which statistically significant changes in Na concentrations for all three plant portions were observed, with leaflets showing a significant decrease (52.56%). However, it has been shown that elevated CO_2 concentrations lead to higher salt tolerances [56,57]. Thus the significant increases in Na concentrations in *S. reticulata* roots and stem may be a plant adaptation to elevated CO_2 levels, in which the species uptakes more of this nutrient, leading to increased plant salt-tolerance.

4.2.1.3. Iron (Fe). Fe concentrations in plants also have also shown inconsistent results when exposed to elevated CO_2 conditions. For example, Fe concentrations in leaves have been both noted to increase [7,33] and decrease [34] under elevated CO_2 conditions, as well as showing no significant differences [39]. In the present study we observed a small, non-significant decrease in Fe concentrations in *S. reticulata* leaflets (1.96%) in plants exposed to higher CO_2 levels, corroborating with the latter report, while Fe concentrations in *S. reticulata* roots decreased significantly in the present study (19.16%), in accordance to studies regarding wheat plants for example [34]. However, stem Fe concentrations also decreased significantly (41.72%), not observed elsewhere. These significant decreases in root and stem could be due to complexation of metals, with sulfhydryl groups being less translocated to the upper regions of the plant [58,59]. As Fe is necessary for chlorophyll synthesis and is required for functioning of a range of enzymes, especially those involved in oxidation and reduction processes, significant Fe decreases could lead to reduced photosynthesis in *S. reticulata* at elevated CO_2 levels.

Overall, the results suggest that variations exist between the uptake and demands for different nutrient elements by *S. reticulata* in response to CO_2 enrichment, because, although several of the nutrients showed lower foliar concentrations in higher CO_2 conditions, such as Na, K, Ca and Mn, consistent with the “nutrient dilution effect”, in some cases increases, as well as decreases, where observed in all three plant portions (Mg and Fe, respectively), while some of the studied nutrients showed no significant changes in some plant portions. However, as plant nutrient stoichiometry is affected by other parameters, such as physiological and biochemical requirements, these regulation processes may inhibit the CO_2 nutrient dilution effect in some cases [60], and climate effects, nutrient availability and soil characteristics also play a role in nutrient apportionment in C3 plants. This means that, overall, it is not possible to predict with certainty what elevated CO_2 effects may cause in *S. reticulata* growth, nutrient apportionment and uptake.

However, the significant differences observed herein due to elevated CO_2 on nutrient concentrations between foliar, stem, and roots, and across all evaluated nutrient, corroborate other studies [61], and will lead to several impacts, both on a micro and macro scale, including litter quality, microbial community alterations, nutrient recycling within eco-systems [61], as well as altered nutrient content, crop yields, and phytoremediation efficiency.

5. Conclusions

Variations were detected between the uptake and demands for different nutrient elements by *S. reticulata* in response to CO_2 enrichment. Na, K, Ca and Mn exhibited lower foliar concentrations in higher CO_2 conditions, consistent with the “nutrient dilution effect”, while increases, as well as decreases, where observed in all three plant portions for Mg and Fe, respectively. However, overall, due to the multi-causal responses in plant nutrient stoichiometry these regulation processes may inhibit the CO_2 nutrient dilution effect. This means that, overall, it is not possible to predict with certainty what elevated CO_2 effects may cause in *S. reticulata* growth, nutrient apportionment and uptake. Biomass, growth increases and certain nutrient levels (Ca, Na) suggest that this species will benefit from elevated CO_2 levels, which may lead to problems due to this species importance as a plague in an economic and pasture management context, while other nutrient levels apportionment changes (Fe, Mn) indicate decreased CO_2 assimilation and photosynthetic rates. Thus, further studies regarding native *S. reticulata* needs for these nutrients should be conducted, for example, with differential soil experimentation, in order to further our understanding of the effects of elevated CO_2 in this plague species, which may display direct effects on food safety, due to altered nutrient content, crop yields, and phytoremediation efficiency.

Conflicts of interest

The authors declare no conflict of interest.

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This study was conducted at a time of great political and economic difficulties in Brazil.

References

[1] M.D. Regan, A.J. Turko, J. Heras, M.K. Andersen, S. Lefevre, T. Wang, M. Bayley, C.J. Brauner, D.T.T. Huong, N.T. Phuong, G.E. Nilsson, Ambient CO2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO2-altered behaviour by taking a hypercapnia dweller down to low CO2 levels, J. Exp. Biol. 219 (2016) 109–118.

[2] IPCC, IPCC: Climate Change 2001: The Scientific Basis.Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 2001.

[3] J.S. Dukes, Will the increasing atmospheric CO2 concentration affect the success of invasive species? in: H.A. Mooney, R.J. Hobbs (Eds.), Invasive Species in a Changing World, Island Press, Washington, 2000.

[4] E.A. Ainsworth, S.P. Long, What have we learned from 15 years of free-air CO2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy, New Phytol. 165 (2) (2005) 351–371.

[5] J.W. Ellsworth, R.A. Harrington, J.H. Fownes, Seedling emergence, growth, and allocation of Oriental bittersweet: effects of seed input, seed bank, and forest floor litter, For. Ecol. Manage. 190 (2-3) (2004) 255–264.

[6] R.W. Pearcy, O. Bjorkman, CO_2 and plants. The response of plants to rising levels of atmospheric carbon dioxide, in: E. Lemon (Ed.), Physiological Effects, Westview Press, Boulder, 1983, pp. 65–105.

[7] S.M. Natali, S.A. Sanudo-Wilhelmy, M.T. Lerdau, Plant and soil mediation of elevated co2 impacts on trace metals, Ecosystems 12 (5) (2009) 715–727.

[8] M.F. Cotrufo, P. Ineson, A. Scott, Elevated CO2 reduces the nitrogen concentration of plant tissues, Glob. Change Biol. 4 (1) (1998) 43–54.

[9] D.R. Taub, B. Miller, H. Allen, Effects of elevated CO2 on the protein concentration of food crops: a meta-analysis, Glob. Change Biol. 14 (3) (2008) 565–575.

[10] S. Ishijima, M. Ohnishi, Regulation of enzyme activities by free Mg2+ concentration regulation of stromal fructose-1,6-biphosphatase and ribulose 1,5-bisphosphate carboxylase activities, J. Appl. Glycosci. 49 (2) (2002) 199–203.

[11] P. Parolin, *Senna reticulata* (Willd.) H. S. Irwin & Barneby (Fabaceae) as “pasture killer” (“matapasto”) pioneer tree in Amazonian floodplains, Ecol. Apl. 4 (2005) 41–46.

[12] P. Parolin, Seed germination and early establishment of 12 tree species from nutrient-rich and nutrient-poor Central Amazonian floodplains, Aquat. Bot. 70 (2) (2001) 89–103.

[13] P. Parolin, L.V. Ferreira, A.L.K.M. Albernaz, S.S. Almeida, Tree species distribution in varzea forests of Brazilian Amazonia, Folia Geobot. 39 (4) (2004) 371–383.

[14] A.A. Iglesias, D.H. González, C.S. Andreo, The C4 pathway og photoshythesis and its regulation, Biochem. Educ. 14 (3) (1985) 98–102.

[15] Z. Tuba, H.K. Lichtenthaler, Long-term acclimation of plants to elevated CO2 and its interaction with stresses, Ann. N. Y. Acad. Sci. 113 (1) (2007) 135–146.

[16] EURACHEM/CITAC, Quantifying uncertainty in analytical measurement, EURACHEM/CITAC Guide, (2000).

[17] J. Kragten, Calculating standard deviations and confidence intervals with a universally applicable spreadsheet technique, Analyst 119 (1994) 2161–2165.

[18] T.S. Madhulatha, An overview on clustering methods, IOSR J. Eng. 2 (3) (2012) 719–725.

[19] Matworks, Neural Network Toolbox User’s Guide: For Use With MATLAB, Matlab6p1, (2009).

[20] J.F. Hair, R.E. Anderson, R.L. Tathan, W.C. Black, Multivariate Data Analysis, 5th ed., Prentice Hall, New Jersey, 1998.

[21] S.J.E. Wand, G.F. Midgley, M.H. Jones, P.S. Curtis, Responses of wild C4 and C3 grass (Poaceae) species to elevated atmospheric CO2 concentration: a meta-analytic test of current theories and perceptions, Glob. Change Biol. 5 (1999) 723–741.

[22] K.P. White, J.A. Langley, D.R. Cahoon, J.P. Megonigal, C3 and C4 biomass allocation responses to elevated CO2 and nitrogen: contrasting resource capture strategies, Estuar. Coasts 35 (2012) 1028–1035.

[23] P.C.D. Newton, Direct effects of increasing carbon dioxide on pasture plants and communities, N. Z. J. Agric. Res. 34 (1) (1991) 1–24.

[24] M.A. Marabesi, Efeito do alto CO2 no crescimento inicial e na fisiologia da fotossíntese em plântulas Senna alata (L.) Roxb. Botanical Institute of the State Secretariat of the Environment, São Paulo, 2007 p. 78.

[25] J.P. Lynch, S.B. St Clair, Mineral stress: the missing link in understanding how global climate change will affect plants in real world soils, Field Crops Res. 90 (1) (2004) 101–115.

[26] A. Gorissen, Elevated CO2 evokes quantitative and qualitative changes in carbon dynamics in a plant/soil system: mechanisms and implications, Plant Soil 187 (1996) 289–298.

[27] P.J. Gregory, J.A. Palta, G.R. Batts, Root systems and root:mass ratio–carbon allocation under current and projected atmospheric conditions in arable crops, Plant Soil 187 (1996) 221–228.

[28] P.S. Curtis, L.M. Balduman, B.G. Drake, D.F. Whigham, Elevated atmospheric Co2 effects on belowground processes in C-3 and C-4 estuarine Marsh communities, Ecology 71 (5) (1990) 2001–2006.

[29] E.A. Paul, R.M.N. Kucey, Carbon flow in plant microbial associations, Science 213 (4506) (1981) 473–474.

[30] E. Ainsworth, P. Davey, C. Bernacchi, O. Dermody, E. Heaton, D. Moore, P. Morgan, S. Naidu, H. Ra, X. Zhu, A meta-analysis of elevated [CO2] effects on soybean (Glycine max) physiology, growth and yield, Glob. Change Biol. 8 (2002) 695–709.

[31] S. Wullschleger, C. Gunderson, P. Hanson, K. Wilson, R. Norby, Sensitivity of stomatal and canopy conductance to elevated CO2 concentration–interacting variables and perspectives of scale, New Phytol. 153 (2002) 485–496.

[32] M.N.V. Prasad, K.S. Sajwan, N. Ravi, Trace Elements in the Environment: Biogeochemistry, Biotechnology and Bioremediation, CRC Press, Boca Raton, 2006.

[33] J.L. Hall, Cellular mechanisms for heavy metal detoxification and tolerance, J. Exp. Bot. 53 (366) (2002) 1–11.

[34] M. Pal, L.S. Rao, A.C. Srivastava, V. Jain, U.K. Sengupta, Impact of CO2 enrichment and variable nitrogen supplies on composition and partitioning of essential nutrients of wheat, Biol. Plant 47 (2) (2003) 227–231.

[35] H.H. Rogers, G.B. Runion, S.V. Krupa, Plant-responses to atmospheric Co2 enrichment with emphasis on roots and the rhizosphere, Environ. Pollut. 83 (1-2) (1994) 155–189.

[36] B. Schaffer, A.W. Whiley, C. Searle, R.J. Nissen, Leaf gas exchange, dry matter partitioning, and mineral element concentrations in mango as influenced by elevated atmospheric carbon dioxide and root restriction, J. Am. Soc. Hortic. Sci. 122 (6) (1997) 849–855.

[37] S.A. Prior, G.B. Runion, H.H. Rogers, H.A. Torbert, Effects of atmospheric CO2 enrichment on crop nutrient dynamics under no-till conditions, J. Plant Nutr. 31 (4) (2008) 758–773.

[38] A. Fangmeier, U. Gruters, P. Hogy, B. Vermehren, H.J. Jager, Effects of elevated CO2 nitrogen supply and tropospheric ozone on spring wheat. 2. Nutrients (N, P, K, S, Ca, Mg, Fe, Mn, Zn), Environ. Pollut. 96 (1997) 43–59.

[39] J. Penuelas, S.B. Idso, A. Ribas, B.A. Kimball, Effects of long-term atmospheric CO2 enrichment on the mineral concentration of Citrus aurantium leaves, New Phytol. 135 (3) (1997) 439–444.

[40] R. Gleadow, W. Foley, I. Woodrow, Enhanced CO2 alters the relationship between photosynthesis and defense in cyanogenic Eucalyptus cladocalyx F. Muell, Plant Cell Environ. 21 (1998) 12–22.

[41] E.P. Hamerlynck, T. Huxman, T. Charlet, S. Smith, Effects of elevated CO2 (FACE) on the functional ecology of the drought-deciduous Mojave Desert shrub, Lycium andersonii, Environ. Exp. Bot. 48 (2002) 93–106.

[42] M. Kubiske, D. Zak, K. Pregitzer, Y. Takeuchi, Photosynthetic acclimation of overstory Populus tremuloides and understory Acer saccharum to elevated atmospheric CO2 concentration: interactions with shade and soil nitrogen, Tree Physiol. 22 (2002) 321–329.

[43] R. Leegood, S. Von Caemmerer, C.P. Osmond, Metabolite transport and photosynthetic regulation in C4 and CAM plants, in: D.T. Dennis, D.H. Turpin, D.D. Layzell, D.K. Lefebvre (Eds.), Plant Metabolism, Longman, Singapore, 1997, pp. 341–369.

[44] X.P. Yi, M. McChargue, S. Laborde, L.K. Frankel, T.M. Bricker, The manganese-stabilizing protein is required for photosystem II assembly/stability and photoautotrophy in higher plants, J. Biol. Chem. 280 (16) (2005) 16170–16174.

[45] A.V. Shitov, O.V. Pobeguts, T.N. Smolova, S.I. Allakhverdiev, V.V. Klimov, Manganese-dependent carboanhydrase activity of photosystem II proteins, Biochemistry-Moscow 74 (5) (2009) 509–517.

[46] T. Youssef, P. Saenger, Photosynthetic gas exchange and accumulation of phytotoxins in mangrove seedlings in response to soil physico-chemical characteristics associated with waterlogging, Tree Physiol. 18 (5) (1998) 317–324.

[47] Q. Li, L.-S. Chen, H.-X. Jiang, N. Tang, L.-T. Yang, Z.-H. Lin, Y. Li, G.-H. Yang, Effects of manganese-excess on CO2 assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase, carbohydrates and photosynthetic electron transport of leaves, and antioxidant systems of leaves and roots in Citrus grandis seedlings, BMC Plant Biol. (2010) 10–42.

[48] P.F. Brownell, C.J. Crosslan, Requirement for sodium as a micronutrient by species having C4 dicarboxylic photosynthetic pathway, Plant Physiol. 49 (5) (1972) 794-&.

[49] A.R. Yeo, T.J. Flowers, The absence of an effect of the Na/Ca ratio on sodium-chloride uptake by rice (Oryza-Sativa-L), New Phytol. 99 (1) (1985) 81–90.

[50] C. Schmidt, T. He, G.R. Cramer, Supplemental calcium does not improve growth of salt-stressed brassicas, Plant Soil 6 (1993) 415–418.

[51] P.A. Essah, Sodium Transport and Accumulation in Arabidopsis thaliana, Cambridge University, Cambridge, UK, 2002.

[52] S. Wang, C. Wan, Y. Wang, H. Chen, Z. Zhou, H. Fu, R. Sosebee, The characteristics of Na+, K+ and free proline distribution in several drought-resistant plants of the Alxa Desert, China, J. Arid Environ. 56 (2004) 525–539.

[53] D. Schachtman, R. Kumar, J. Schroeder, E. Marsh, Molecular and functional characterization of a novel lowaffinity cation transporter (LCT1) in higher plants, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11079–11084.

[54] J.C. Melgar, M. Benlloch, R. Fernandez-Escobar, Calcium increases sodium exclusion in olive plants, Sci. Hortic. 109 (3) (2006) 303–305.

[55] J.L. Zhang, T.J. Flowers, S.M. Wang, Mechanisms of sodium uptake by roots of higher plants, Plant Soil 326 (1-2) (2010) 45–60.

[56] O. Urban, Physiological impacts of elevated CO2 concentration ranging from molecular to whole plant responses, Photosynthetica 41 (1) (2003) 9–20.

[57] J.Y. Li, X.H. Liu, Q.S. Cai, H. Gu, S.S. Zhang, Y.Y. Wu, C.J. Wang, Effects of elevated CO2 on growth, carbon assimilation, photosynthate accumulation and related enzymes in rice leaves during sink-source transition, J. Integr. Plant Biol. 50 (2008) 723–732.

[58] J.H. Rodriguez, A. Klumpp, A. Fangmeier, M.L. Pignata, Effects of elevated CO2 concentrations and fly ash amended soils on trace element accumulation and translocation among roots, stems and seeds of Glycine max (L.) Merr, J. Hazard. Mater. 187 (1–3) (2011) 58–66.

[59] S. Dwivedi, R.D. Tripathi, S. Srivastava, S. Mishra, M.K. Shukla, K.K. Tiwari, R. Singh, U.N. Rai, Growth performance and biochemical responses of three rice (Oryza sativa L.) cultivars in fly-ash amended soil grown, Chemosphere 67 (1) (2007) 140–151.

[60] D.R. Taub, X.Z. Wang, Why are nitrogen concentrations in plant tissues lower under elevated CO2? A critical examination of the hypotheses, J. Integr. Plant Biol. 50 (11) (2008) 1365–1374.

[61] B.D. Duval, J.C. Blankinship, P. Dijkstra, B.A. Hungate, CO2 effects on plant nutrient concentration depend on plant functional group and available nitrogen: a meta-analysis, Plant Ecol. 213 (3) (2012) 505–521.